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Original Paper

Lipopolysaccharides (LPS) Induced Angiogenesis During Chicken Embryogenesis is Abolished by Combined ETA/ETB Receptor Blockade

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Key Words

Lipopolysaccharides (LPS) • Angiogenesis • Chicken chorioallantoic membrane (CAM) • Endothelin (ET)

Abstract

Background/Aims: Angiogenesis plays a key role during embryonic development. The vascular endothelin (ET) system is involved in the regulation of angiogenesis. Lipopolysaccharides (LPS) could induce angiogenesis. The effects of ET blockers on baseline and LPS-stimulated angiogenesis during embryonic development remain unknown so far. **Methods:** The blood vessel density (BVD) of chorioallantoic membranes (CAMs), which were treated with saline (control), LPS, and/or BQ123 and the ETB blocker BQ788, were quantified and analyzed using an IPP 6.0 image analysis program. Moreover, the expressions of ET-1, ET-2, ET3, ET receptor A (ETRA), ET receptor B (ETRB) and VEGFR2 mRNA during embryogenesis were analyzed by semi-quantitative RT-PCR. **Results:** All components of the ET system are detectable during chicken embryogenesis. LPS increased angiogenesis substantially. This process was completely blocked by the treatment of a combination of the ETA receptor blockers-BQ123 and the ETB receptor blocker BQ788. This effect was accompanied by a decrease in ETRA, ETRB, and VEGFR2 gene expression. However, the baseline angiogenesis was not affected by combined ETA/ETB receptor blockade. **Conclusion:** During chicken embryogenesis, the LPS-stimulated angiogenesis, but not baseline angiogenesis, is sensitive to combined ETA/ETB receptor blockade.

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Introduction

Endothelin-1 (ET-1) has been shown to play a key role in the pathogenesis of cardiac and renal diseases [1-5]. It is elevated in patients with essential hypertension [6] and in particular pregnant women with pregnancy induced hypertension [7]. Besides its role in renal and cardiovascular diseases, ET-1 might play a role in angiogenesis during embryonic development as well, since it is known that ET-1 might interact with factors critical for angiogenesis during embryonic development. In the current study, we analyzed the time-dependent mRNA expression of components of the ET system in the whole chicken embryos, and in the chicken chorioallantoic membranes (CAMs), as well as the effects of blocking both ET receptors on LPS-stimulated angiogenesis in CAMs.

Materials and Methods

Assessment of angiogenesis using CAMs

Chicken eggs (obtained from the Avian Farm of the South China Agriculture University) were incubated for 5, 7, and 9 days respectively, to analyze the mRNA expression of ET1, ET2 and ET3 as well as ET receptors and VEGFR2. The 7-day CAMs were treated with LPS (10 or 100 µg/ml). A combined ETA and ETB receptor blockade was achieved by simultaneously treating the eggs with BQ123 and BQ788 (1, 5 or 10 µg/ml respectively) or saline (control) for 48 hours. All surviving embryos were harvested for analysis. The CAMs and accompanying blood vessels in the control, BQ123 and BQ788 or/and LPS-treated groups were photographed using a Canon Powershot SX130 IS digital camera (12.1M Pixels). The color pictures were turned to grayscale pictures by Photoshop.

Semiquantitative RT-PCR

Total RNA was isolated from the gastrula chicken embryos or chicken CAM tissues using a Trizol kit (Invitrogen, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized to a final volume of 25 µl using SuperScript RIII first-strand (Invitrogen, USA). Following reverse transcription, PCR amplification of the cDNA was performed. The sets of primers used for semi-quantitative RT-PCR are provided in the Table 1. The PCR reactions were performed in a Bio-Rad S1000TM Thermal cycler (Bio-Rad, USA). The final reaction volume was 50 µl composed of 1 µl of first-strand cDNA, 25 µM forward primer, 25 µM reverse primer, 10 µl PrimeSTART™ Buffer (Mg2⁺ plus), 4 µl dNTPs Mixture (TaKaRa, Japan), 0.5 µl PrimeSTART™ HS DNA Polymerase (2.5 U/µl TaKaRa, Japan), and RNase-free water. cDNA was amplified for 30 cycles. One round of amplification was performed at 94°C for 30 s, 30 s at 58°C, and 30 s at 72°C. The PCR products (20 µl) were resolved using 1% agarose gels (Biowest, Spain) in 1× TAE buffer (0.04 M Trisacetate and 0.001 M EDTA) and 10, 000× GeneGreen Nucleic Acid Dye (TIANGEN, China) solution. The resolved products were visualized using a transilluminator (SYNGENE, UK), and photographed using a computer-assisted gel documentation system (SYNGENE, UK). All experiments were replicated at least three times.

Data analysis

The blood vessel density (BVD) were quantified and analyzed as follows. The areas occupied by the blood vessel plexus were quantified using an IPP 6.0 image analysis program. The BVD was expressed as the percentage of area occupied by the blood vessel over the whole area under the microscopic field as previously described [8-11]. Statistical analysis

Table 1. Primers sets used in the RT-PCR analysis

Primers	Forward	Reverse
ET-1	GGAGCTGTTTACCCCCACTC	GTGCCCTTTTAAACGGGGAGA
ET-2	GAGCTGTCCAAGTCAGACGC	TCAGCCCAAGTGCCCTTTTA
ET-3	GGAGTGCCTCTACTACTGCC	GCAAACGTTAAACCTTCTTCTGTC
ETRA	ACTAGACGGCCTCCGGTATC	TCGGGCCATTCTCATACAC
ETRB	GTGTCATGCCTGGTGTTCGT	GCCAGTCCTCTGCAAGTAGC
VEGFR2	TTGGCTGGCGGTATTCACAT	GTCTCTGGAGGAAAGCACCC
GAPDH	GTCAACGGATTGGCCGTAT	AATGCCAAAGTTGTCATGGATG

of all the experimental data generated was performed using a SPSS 13.0 statistical package program for Windows. The data were presented as mean \pm SE. Statistical significance were determined using one-way analysis of variance (ANOVA). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate significant difference between control and experimental groups.

Results

Endothelin (ET) signaling is involved in the chicken CAM development

We detected the mRNA expression of all ligands of the ET system in the developing chicken embryos (Fig. 1A) at embryonic 5-day (E5.0), 7-day (E7.0) and 9-day (E9.0). However, the expression level varied at different developmental stages (ET1/E5.0, E7.0, E9.0: 15.10, 14.98, 14.88; ET2/ E5.0, E7.0, E9.0: 10.16, 10.18, 10.46; ET3/ E5.0, E7.0, E9.0: 15.28, 15.12, 15.00; ET receptor A (ETRA)/ E5.0, E7.0, E9.0: 5.19, 5.5, 5.26; ET receptor B (ETRB)/ E5.0, E7.0, E9.0: 6.6, 6.29, 6.38; Fig. 1B), which was revealed using quantitative PCR. We further compared the expression of the components of the ET system with the predominant gene involved in angiogenesis, VEGF receptor 2 (VEGFR2/ E5.0, E7.0, E9.0: 12.42, 12.06, 12.25). This analysis showed that ETRB, ET-2 and ET-3 gene expression showed similar time-dependent expression patterns as compared to VEGFR2 in chicken CAMs.

Blocking ET signaling does not significantly affect non-stimulated baseline angiogenesis in chicken CAMs

Using different dosages of a combination of both ETRA and ETRB antagonists (BQ123 and BQ788), we investigated whether or not blocking ET signaling could affect baseline angiogenesis (Fig. 2A). A combination of 1, 5 or 10 $\mu\text{g/ml}$ BQ123 and BQ788 did not affect the BVD of chicken CAMs (Figs. 2B-E; Control = $13.57 \pm 0.91\%$, $N = 4$; BQ123 + BQ788 (1 $\mu\text{g/ml}$) = $14.06 \pm 0.61\%$, $N = 4$; BQ123 + BQ788 (5 $\mu\text{g/ml}$) = $15.95 \pm 0.36\%$, $N = 4$; BQ123 + BQ788 (10 $\mu\text{g/ml}$) = $14.19 \pm 1.34\%$, $N = 4$; Fig. 2F). We further detected the expression of ETRA, ETRB and VEGFR2 mRNA levels in the presence of BQ123 and BQ788 exposed to chicken CAM using quantitative PCR (Figs. 2G-I). The results showed that BQ123 and BQ788 could significantly inhibit the ETRA and ETRB gene expression on chicken CAMs (Control = 1.00 ± 0.11 , $N = 3$; BQ123 + BQ788 (1 $\mu\text{g/ml}$) = 0.62 ± 0.08 , $N = 3$; BQ123 + BQ788 (5 $\mu\text{g/ml}$) = 0.20 ± 0.03 , $N = 3$; BQ123 + BQ788 (10 $\mu\text{g/ml}$) = 0.22 ± 0.02 , $N = 3$; Fig. 2G; Control = 1.00 ± 0.10 , $N = 3$; BQ123 + BQ788 (1 $\mu\text{g/ml}$) = 0.14 ± 0.03 , $N = 3$; BQ123 + BQ788 (5 $\mu\text{g/ml}$) = 0.33 ± 0.04 , $N = 3$; BQ123 + BQ788 (10 $\mu\text{g/ml}$) = 0.27 ± 0.02 , $N = 3$; Fig. 2H). However, the VEGFR2 gene expression did not change significantly (Control = 1.000 ± 0.02082 , $N = 3$; BQ123 + BQ788 (1 $\mu\text{g/ml}$) = 0.8067 ± 0.08686 , $N = 3$; BQ123 + BQ788 (5 $\mu\text{g/ml}$) = 1.169 ± 0.09599 , $N = 3$; BQ123 + BQ788 (10 $\mu\text{g/ml}$) = 1.453 ± 0.01863 , $N = 3$; Fig. 2I).

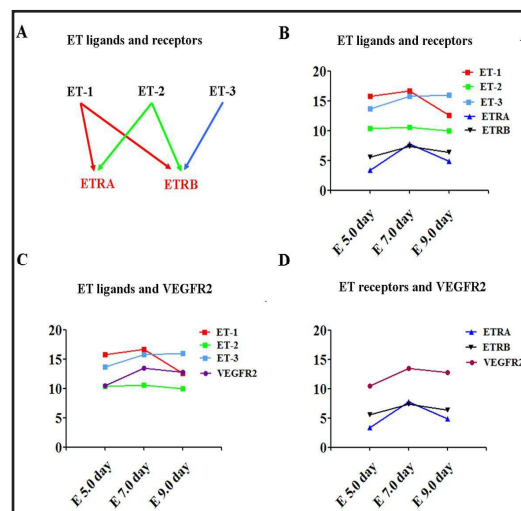


Fig. 1. Assessing the expression patterns of ET ligands and receptors in chicken CAMs. A: The sketches illustrate the variety of ET and its receptors. B-D: Quantitative PCR data showing gene expressions of ET ligands and receptors (B), ET ligands and VEGFR2 (C), ET receptors and VEGFR2 (D) at mRNA level in E5.0, E7.0, E9.0 chicken CAMs. Gene expression is shown as relative expression in relationship to the expression of GAPDH.

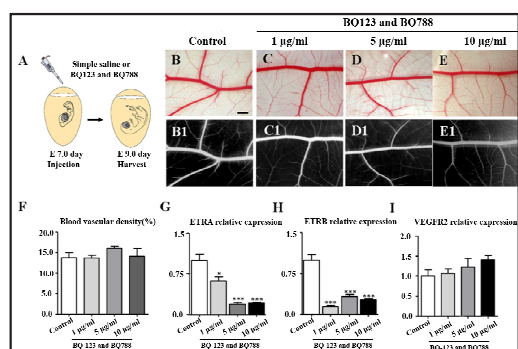


Fig. 2. The assessment of angiogenesis in chick CAM following blockage of ET receptors with antagonists. A: The sketches illustrate treatment of embryos with saline (Control) and BQ123 and BQ788. B-E1: The representative bright-field image of angiogenesis in chicken CAMs exposed to saline (Control, B), 1 µg/ml (C), 5 µg/ml (D), 10 µg/ml (E) of a combination of BQ123 + BQ788, respectively. B1-E1 are representative grayscale pictures. F: The bar graphs showing the comparison of BVD (%) in chicken CAMs between Control and various concentrations of BQ123 + BQ788 groups. G-I: The quantitative PCR data show mRNA expressions of ETRA (G), ETRB (H) and VEGFR2 (I) in Control and various concentrations of BQ123 + BQ788 groups. * $p < 0.05$ and *** $p < 0.001$ indicate significant difference between Control and experimental groups. Scale bars = 1 mm in B-E and in B-E1. ETRA, ETRB and VEGFR2 gene expression is shown as relative expression in relationship to the expression of GAPDH.

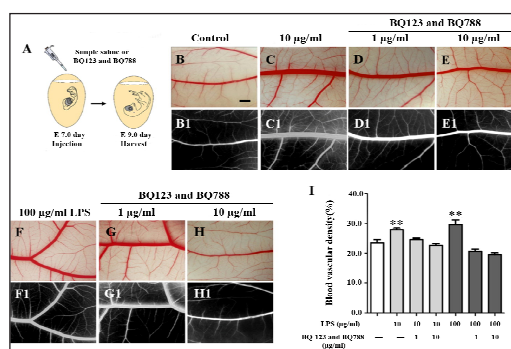


Fig. 3. The assessment of angiogenesis in chicken CAMs in absence/presence of LPS following blockage of ET receptors with antagonists. A: The sketches illustrate results of treating embryos with saline (Control) and/or LPS and/or BQ123+BQ788. B-H1: The representative bright-field images of angiogenesis on chicken CAMs exposed to simple saline (Control, B) and various concentrations of LPS or/and BQ123 + BQ788. B1-H1 are the representative grayscale pictures. I: The bar graphs showing the comparison of BVD (%) of chicken CAMs between Control and various concentrations of LPS or/and BQ123 + BQ788 groups. ** $p < 0.01$ and *** $p < 0.001$ indicate significant difference between Control and experimental groups. Scale bars = 1 mm in B-F and in H-M.

ET signaling is involved in LPS-stimulated angiogenesis on chicken CAMs

We further investigated whether or not blocking ET signaling with BQ123 and BQ788 could affect LPS-induced angiogenesis in chicken CAMs. LPS increased in a dose dependent manner angiogenesis in chick CAM (Figs. 3A-C, F; Control = $23.43 \pm 1.14\%$, $N = 5$; LPS (10 µg/ml) = $27.86 \pm 0.53\%$, $N = 5$; LPS (10 µg/ml) + (BQ123 + BQ788 1 µg/ml) = $24.55 \pm 0.50\%$, $N = 5$; LPS (10 µg/ml) + (BQ123 + BQ788 10 µg/ml) = $22.63 \pm 0.56\%$, $N = 5$; LPS (100 µg/ml) + (BQ123 + BQ788 1 µg/ml) = $20.60 \pm 0.69\%$, $N = 5$; LPS (100 µg/ml) + (BQ123 + BQ788 10 µg/ml) = $19.43 \pm 0.72\%$, $N = 5$; Fig. 3). Blocking ET signaling with two dosages of a combination of BQ123 and BQ788 normalized LPS-induced angiogenesis to baseline levels (Figs. 3B-I).

We also analyzed mRNA expressions of ETRA, ETRB and VEGFR2 in presence of LPS and/or BQ123 and BQ788 (Fig. 4). The results showed that 10 or 100 µg/ml LPS enhanced the expressions of ETRA, ETRB and VEGFR2 in a dose dependent manner (Control = 1.00 ± 0.09 , $N = 3$; LPS (10 µg/ml) = 2.53 ± 0.19 , $N = 3$; LPS (10 µg/ml) + (BQ123 + BQ788 1 µg/ml) = 1.68 ± 0.04 , $N = 3$; LPS (10 µg/ml) + (BQ123 + BQ788 10 µg/ml) = 0.76 ± 0.01 , $N = 3$; LPS (100 µg/ml) = 3.98 ± 0.05 , $N = 3$; LPS (100 µg/ml) + (BQ123 + BQ788 1 µg/ml) = 1.70 ± 0.08 , $N = 3$; LPS (100 µg/ml) + (BQ123 + BQ788 10 µg/ml) = 1.70 ± 0.08 , $N = 3$; Fig. 4A; Control = 1.00 ± 0.02 , $N = 3$; LPS (10 µg/ml) = 2.41 ± 0.12 , $N = 3$; LPS (10 µg/ml) + (BQ123 + BQ788 1 µg/ml) = 2.41 ± 0.12 , $N = 3$; LPS (10 µg/ml) + (BQ123 + BQ788 10 µg/ml) = 0.62 ± 0.09 , $N = 3$; LPS (100 µg/ml) = 4.98 ± 0.18 , $N = 3$; LPS (100 µg/ml) + (BQ123 + BQ788 1 µg/ml) = 1.83

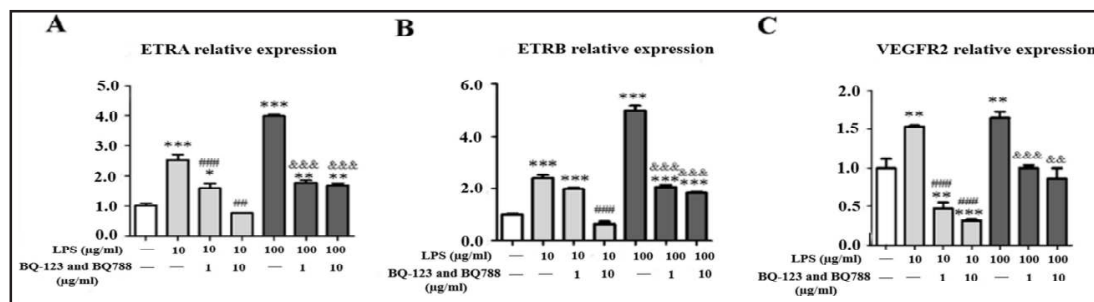


Fig. 4. Analysis of ETRA, ETRB and VEGFR2 mRNA in chicken CAMs in absence/presence of LPS following blockage of both ET receptor subtypes. A-C: The quantitative PCR data show the gene expressions of the ETRA (A), ETRB (B) and VEGFR2 (C) in each group. ** $p < 0.01$ and *** $p < 0.001$ indicate significant difference between Control and experimental groups; ## $p < 0.01$ and ### $p < 0.001$ indicate significant difference between 10 µg/ml LPS and 10 µg/ml LPS + BQ123 + BQ788 treatment groups; && $p < 0.01$ and &&& $p < 0.001$ indicate significant difference between 100 µg/ml LPS and 100 µg/ml LPS + BQ123 + BQ788 treatment groups. ETRA, ETRB and VEGFR2 gene expression is shown as relative expression in relationship to the expression of GAPDH.

± 0.04 , $N = 3$; LPS (100 µg/ml) + (BQ123 + BQ788 10 µg/ml) = 2.07 ± 0.08 , $N = 3$; Fig. 4B; Control = 1.00 ± 0.12 , $N = 3$; LPS (10 µg/ml) = 1.54 ± 0.01 , $N = 3$; LPS (10 µg/ml) + (BQ123 + BQ788 1 µg/ml) = 0.47 ± 0.08 , $N = 3$; LPS (10 µg/ml) + (BQ123 + BQ788 10 µg/ml) = 0.31 ± 0.02 , $N = 3$; LPS (100 µg/ml) = 1.64 ± 0.08 , $N = 3$; LPS (100 µg/ml) + (BQ123 + BQ788 1 µg/ml) = 1.00 ± 0.04 , $N = 3$; LPS (100 µg/ml) + (BQ123 + BQ788 10 µg/ml) = 0.86 ± 0.14 , $N = 3$; Fig. 4C). Interestingly, both 1 µg/ml and 10 µg/ml of BQ123 and BQ788 administration could significantly suppress the LPS-induced expressions of ETRA and ETRB (Figs. 4A-C).

Discussion

The current study demonstrated that all components of the ET system are detectable during chicken embryonic development. The signal for ET-1 mRNA was most prominent and the signal for ETRA was relatively low as compared to the expression of VEGFR2, a maker of angiogenesis [12-14]. Combined blockage of ET receptors using classical ETRA and ETRB blockers did not affect baseline angiogenesis and the mRNA expression of the angiogenesis marker VEGFR2, whereas ETRA and ETRB mRNA expression were significantly decreased after combined ETRA/ETRB blockade. LPS increased angiogenesis substantially. The elevation of LPS-induced angiogenesis could be completely blocked by the treatment with a combination of the ETRA blocker - BQ123 and ETRB blocker - BQ788. This effect was accompanied by decreased expression of ETRA, ETRB, and VEGFR2. Our study did not prove that this decrease in the VEGFR2 gene expression was caused by a decreased ETRA and ETRB mRNA expression. VEGFR2 expression works simply as a marker of angiogenesis. This interpretation is consistent with all data obtained in our study. Alteration of VEGFR2 gene expression parallels to angiogenesis, which was known from previous studies. The underlying molecular pathways, which could explain the effects of combined ET receptor blockade on LPS-induced angiogenesis in contrast to no effects of combined ET receptor blockade on baseline angiogenesis in chicken embryos, remain to be elucidated.

The effect of ET-1 on angiogenesis in CAM has been well established [15-20]. However, to the best of our knowledge so far, there is no study showing that the LPS-induced angiogenesis in CAM can be blocked by the combined application of the ETA blocker BQ123 and the ETB blocker BQ 788.

In any case, it is of note that the combined blockade of the ET receptors causes a downregulation of the ETRA and ETRB mRNA expression under baseline and LPS stimulation. This is remarkable, since a pharmacological blockade of receptors usually

causes a compensatory upregulation of the receptor mRNA and protein [21]. Again, the underlying molecular pathways explaining this unusual observation needs to be addressed in further studies.

It is a clear limitation of the current study, that only a combination of BQ123 and BQ788 was investigated. It is thus not possible to separate potential ETA specific effects from ETB mediated effects on baseline and LPS-stimulated angiogenesis.

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Disclosure Statement

The authors have declared that no competing interests exist.

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